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Mild Dyslipidemia in Mice following Targeted Inactivation of the Hepatic Lipase Gene*

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In order to gain better understanding of the function of hepatic lipase (HL) *in vivo*, we have generated mice that lack HL using gene targeting in embryonic stem cells. No mRNA for HL was detected in the liver of homozygous mutants, and no HL activity was detected in their plasma. Total cholesterol levels in plasma of mutant mice were increased by about 30% compared with wild type animals. Plasma phospholipids and high density lipoprotein (HDL) cholesterol were also increased, but plasma levels of triglycerides were not altered. Analysis of density fractions of plasma lipoproteins revealed that HDL_1 (d = 1.02–1.04) was increased in homozygous mutants fed regular chow. In response to a diet containing high fat and high cholesterol, HDL cholesterol was doubled in the mutants, but was slightly decreased in the wild type mice. These results clearly demonstrate the importance of HL in HDL remodeling and metabolism *in vivo*. Various earlier studies suggested a role of HL in metabolism of triglyceride-rich particles, but the mutant mice appear to have no impairment in clearing them; the mutants clear exogenously introduced chylomicrons from plasma at a normal rate, and they tolerate acute fat loading as well as normal animals unless the loading is extreme. These differences may reflect species differences. However, it is also possible that the consequence of *absence of HL* as in our mutants is different from the consequence when *nonfunctional HL protein is present* as in the human HL-deficient patients and in rats treated with HL antibodies. We hypothesize that absence of HL in mutant mice allows other lipases to bind to the sites in the liver normally occupied by HL and facilitate the clearance of triglyceride-rich particles in these mice.

Hepatic lipase (HL)[1] is synthesized by hepatocytes and following secretion it binds to the surface of the sinusoidal endothelium of the liver (1). Several lines of evidence suggest that HL plays an important role in high density lipoprotein (HDL) metabolism. Specifically, HL is thought to be responsible for the conversion of HDL_2 to HDL_3. HL activity reduces the concentration of phospholipids and triglycerides in the HDL_2 subfraction and increases the phospholipid concentration in the HDL_3 subfraction (2), and Kuusi *et al.* (3) have reported that HL activity is negatively correlated with the level of HDL_2 cholesterol, HDL_2 phospholipid, HDL_2 protein, and total triglyceride. Hepatic lipase deficiency in humans results in the accumulation of HDL particles containing elevated levels of triglyceride and/or phospholipid (4–7). In experimental animals treated with antibodies blocking the function of HL, the HDL_2 subfraction increases in triglyceride and/or phospholipid content, whereas the HDL_3 subfraction is unaffected (1, 8–10). The observation that HDL_2 phospholipids are substrate for HL *in vitro* (11, 12) further supports the suggestion that HL is involved in the hydrolysis of phospholipids in HDL_2.

Studies have shown that hepatic lipase enhances the uptake of cholesterol by hepatocytes *in vitro* (13, 14). *In vivo*, inhibition of HL activity by HL antibody administration significantly decreases the rate of chylomicron remnant cholesterol uptake by the liver (15, 16). HL has been implicated in the metabolism of VLDLs, intermediate density lipoproteins (IDL), and low density lipoproteins (LDL); administration of HL antiserum can increase the triglyceride, phospholipid and cholesterol content of these lipoproteins (8, 9, 17, 18). Similarly, in some human individuals with HL deficiency, triglycerides and phospholipids accumulate in LDL and VLDL (5, 19, 20).

Although HL is clearly involved in the metabolism of circulating lipoproteins, inconsistencies among various investigations prevent a definite conclusion as to its specific physiological role. Some of these inconsistencies may be due to differences in experimental procedures. Others may be due to inadequacies in the experimental model. For example, results from humans naturally deficient in HL may be confounded by the co-existence of other abnormalities that contribute to the observed phenotype (20, 7). Results of HL inactivation by antibody treatment are variable partly because antibody treatment does not always completely inhibit the enzyme and partly because function of HL as a facilitator of remnant uptake may well be independent of catalytic activity of HL. Specific and complete inactivation of HL in mice by genetic means should supply an useful model for enhancing our understanding of the function of HL *in vivo*. Accordingly, we have used gene target-

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Mutant mice lacking hepatic lipase have been deposited in and may be obtained from the Induced Mutant Resource, Jackson Laboratory, Bar Harbor, ME.

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[1] The abbreviations used are: HL, hepatic lipase; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; ES cells, embryonic stem cells; LPL, lipoprotein lipase; *neo*, neomycin resistance gene; TK, thymidine kinase gene; apo, apolipoprotein; kb, kilobase pair(s).

ing in mouse embryonic stem cells to generate mice that specifically lack HL, and we have described the consequences of HL deficiency on the lipoprotein metabolism in the resulting animals.

EXPERIMENTAL PROCEDURES

Production of Targeting Constructs—Strain 129 mouse DNA was completely digested with *Eco*RI and ligated to the arms of λ phage Charon 32. From a phage library made with this DNA, a clone containing a 7.3-kb *Eco*RI fragment having sequences homologous to exons 4–6 of the rat HL sequence was isolated using a rat HL cDNA as a probe (21).

The positive-negative selection system of Mansour *et al.* (22) was used to disrupt the HL gene. HL targeting constructs were made by inserting an *Xho*I/*Hin*dIII fragment containing the bacterial neomycin resistance (*neo*) gene driven by the herpes simplex thymidine kinase (TK) promoter and polyoma virus enhancer (pMC1neopoly(A), Stratagene, La Jolla, CA) into an *Nco*I site of exon 4 within the 7.3-kb *Eco*RI fragment by blunt end ligation (see Fig. 1). Two constructs, HL1 and HL2, were made that differ in the orientation of the *neo* gene with respect to the HL gene. The *neo* gene is in the opposite transcriptional orientation in HL1 and in the same orientation in HL2. In addition, the TK gene with a polyoma virus enhancer was inserted both 5′ and 3′ of the region of homology of the HL gene, that are 0.25 and 7 kb in length, respectively (see Fig. 1). The targeting constructs were linearized at the unique *Sac*II site prior to introduction into ES cells.

Targeting of the HL Locus in Mouse Embryonic Stem Cells—The targeting constructs were individually electroporated into the mouse ES cell line, E14TG2a (23), as described by Piedrahita *et al.* (24). In each experiment, 2–3 × 10^7 ES cells were electroporated with 2 nmol of CsCl-purified targeting DNA. Following electroporation, cells were cultured for approximately 24 h in ES cell medium and then in that contained G418 (150–200 μg/ml) and ganciclovir (2 μM). Following 10–14 days of selection, surviving ES cell colonies were individually picked and expanded. An aliquot of each colony was frozen, and the remainder was used to prepare genomic DNA. Correctly targeted cells were identified by Southern blot analysis following digestion of DNA with *Hin*dIII, *Bgl*II, *Nco*I, *Xba*I, and *Bam*HI with a 950-base pair *Eco*RI/*Bam*HI fragment that includes exon 4 of the mouse HL gene as a probe (see Fig. 1).

Production of Mice with Modified Alleles of HL—A total of 222 blastocysts were injected with a targeted cell line to produce chimeric mice as described (25). Following transfer to pseudopregnant recipient females, 36 pups were born, 30 survived to weaning, 21 were chimeric. Five of 6 male chimeras and 3 of 15 female chimeras transmitted the ES cell genome through the germ line after mating with C57BL/6J mice. Offspring that inherited the embryonic stem cell genome as determined by coat color were screened for the presence of the modified HL allele by genomic Southern hybridization analysis as described above for ES cell DNA analysis. Interbreeding of heterozygous offspring was used to produce mice homozygous for the modified HL allele. Chimeras were also mated with 129 females to obtain mutants in having the inbred 129 genetic background.

Mice were fed *ad libitum* either regular chow (containing 4.5% (w/w) fat and 0.022% (w/w) cholesterol (number 5012, Ralston Purina, St. Louis, MO)) or an atherogenic diet (containing 15.8% (w/w) fat, 1.25% (w/w) cholesterol, and 0.5% (w/w) sodium cholate (TD88051, Teklad Premier, Madison, WI)). The animals were handled following the National Institutes of Health guidelines for the care and use of experimental animals.

Analysis of Plasma Lipids, Apolipoproteins, Lipoproteins, and Hepatic Lipase—Following an overnight fast, 200–400 μl of blood were collected by retro-orbital bleeding into tubes containing 0.3 mg of EDTA, 25 mg of gentamicin sulfate, and 11.5 milliunits of aprotinin (Sigma). Plasma was collected by centrifugation at 8,000 × *g* for 10 min at 4 °C. Agarose gel electrophoresis of whole plasma, and determination of total cholesterol, HDL cholesterol, and triglyceride were performed as described previously using reagents from Sigma (26, 27). Plasma levels of phospholipids were determined by the method of Yoshida *et al.* (28). Plasma, frozen at −80 °C, was used for HL activity determination using emulsified triolein in 2 M NaCl (29); no other plasma lipase is active under these conditions. Activity for each sample was determined at two concentrations to ascertain linearity in the assay. Student's *t* test of unpaired observations was used to determine statistical significance.

For lipoprotein analysis, blood was also collected for lipoprotein analysis in the morning from nonfasted mice by heart puncture under anesthesia with a lethal dose of avertin (2,2,2,-tribromoethanol). Female mice of mixed genetic background between C57BL/6J and 129, and of similar age (8–10 months old), were fed regular chow (six each genotype) or fed an atherogenic diet for two weeks (four each genotype). Lipoproteins were fractionated from 1 ml of plasma combined from two to three mice by sequential density ultracentrifugation at 100,000 rpm at 4 °C (30). Lipoproteins in the density ranges of $d < 1.006$, $d = 1.006–1.02$, $d = 1.02–1.04$, $d = 1.04–1.06$, $d = 1.06–1.08$, and $d = 1.08–1.10$ g/ml were obtained following successive centrifugation for 2.5 h. The $d = 1.10–1.21$ g/ml fraction was isolated following centrifugation for 4 h. Lipoproteins from each fraction were recovered by tube slicing and dialyzed in 10 mM Tris buffer, pH 7.4, 150 mM NaCl, and 1 mM EDTA. Lipoproteins were separated by agarose gel electrophoresis and detected by Fat Red 7B (Ciba Corning, Palo Alto, CA). The apolipoproteins on agarose gels were transferred to nitrocellulose by blotting and were detected by immunoreaction with specific antibodies against human apoB, human apoA-I, and rat apoE (30). The apolipoprotein composition of each fraction was determined by SDS-polyacrylamide gel electrophoresis of each fraction followed by Coomassie Brilliant Blue staining of the proteins. Lipid analyses of total plasma and of fractions were carried out as described previously (30) using reagents for cholesterol and triglycerides from Boehringer Mannheim and for phospholipids from Wako Chemical USA Inc. (Richmond, VA).

RNA Isolation and Analysis—Total RNA was isolated from liver according to Chomczynski and Sacchi (31) using RNAzol™B (Tel-test, Friendswood, TX). Poly(A) RNA was prepared as described by Kingston *et al.* (32). Northern blot analysis of poly(A) RNA was done in a 1.2% agarose gel containing 2.2 M formaldehyde. RNA was transferred to nylon membranes (Hybond-N, Amersham Corp.) and was hybridized to the rat hepatic lipase cDNA probe (21). Human β-actin (Clontech, Palo Alto, CA) was used as an internal standard to normalize loadings.

Chylomicron Clearance—The mesenteric ducts of wild type mice were cannulated, and the animals were then fed by stomach tube a bolus of 0.3 ml of corn oil containing 100 μCi of [1,2-^{3}H]cholesterol (Amersham). Lymph was collected overnight in the absence of preservatives. ^{3}H-Labeled chylomicrons (approximately 0.8 mg of cholesteryl ester/100 mg of triglycerides) were collected by centrifuging in SW 55 Ti rotor (Beckman Instruments) at 28,000 rpm for 1 h. Eight-month-old male mice homozygous for the inactivated HL gene and age-matched control male mice were anesthetized with Nembutal intraperitoneally, and 5.0 mg of chylomicron triglyceride in 0.15 ml of saline were injected through the femoral vein. At specified times there after, 50-μl blood samples were collected from the tail vein into heparinized capillary tubes. Radioactivity in total plasma volume was calculated by assuming a blood volume of 5.5% body weight (33). The livers were perfused for 30 s with saline through the inferior cava vein, in order to remove residual blood, and processed for determination of incorporated radioactivity as described previously (34).

Fat Tolerance Test—Five-month-old male mice fasted for 16 h with free access to 10% fructose as a drinking solution were lightly anesthetized by Metofane, and olive oil (0.4 or 1 ml, Filippo Berioo, Lucca, Italy) was administered intragastrically as a bolus. Approximately 50 μl of blood were collected retro-orbitally to measure plasma triglyceride levels at 0, 2, 3, 4, 5, 6, and 7 h after the loading.

RESULTS

Targeting of the Hepatic Lipase Locus in Mouse Embryonic Stem Cells—We made targeting constructs using the *Eco*RI fragment that contains exons 4–6 of the mouse HL gene. We confirmed by nucleotide sequencing that exon 4 is 118 base pairs and is 91% identical to rat HL exon 4 and 81% identical to human HL exon 4 at the nucleotide level. Since exon 4 is thought to contain sequences important for biological activity (35, 36), we inserted a selectable marker gene into this exon to disrupt the HL gene and completely eliminate HL activity (Fig. 1). Four electroporation experiments were carried out with each of the two targeting constructs, HL1 and HL2. From these experiments, 248 doubly resistant colonies were analyzed by Southern blotting for gene targeting at the HL locus, and five independent and correctly targeted cell lines were identified. Three out of 125 colonies screened were correctly targeted using the vector HL1 containing the *neo* gene in the opposite transcriptional orientation with respect to the HL gene, and 2 out of 123 were targeted using the HL2 vector containing the *neo* gene in the same orientation. Thus although the numbers

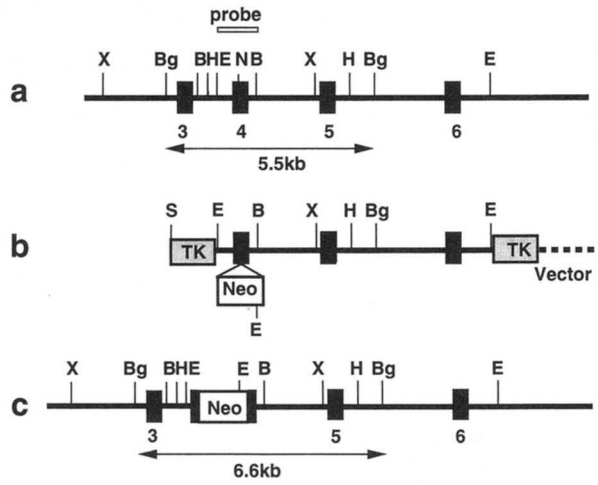

FIG. 1. **Targeted disruption of the mouse hepatic lipase gene.** *a*, the mouse hepatic lipase gene. Exons 3–6 are indicated by *black boxes*. *b*, the targeting construct. The neomycin resistant gene (*neo*) was inserted into an *Nco*I (*N*) site of exon 4. The herpes simplex thymidine kinase gene (*TK*) sequence was attached to both ends of the region of homology, and the plasmid was linearized at the *Sac*II site (*S*) prior to electroporation. *c*, modified locus can be detected by Southern blot analysis using the probe shown in *a*. Restriction enzyme sites used for the analyses are shown: *X*, *Xba*I; *Bg*, *Bgl*II; *B*, *Bam*HI; *H*, *Hin*dIII; *E*, *Eco*RI. The 5.5-kb *Bgl*II fragment of the endogenous locus altered to 6.6 kb in the targeted locus is shown as an example.

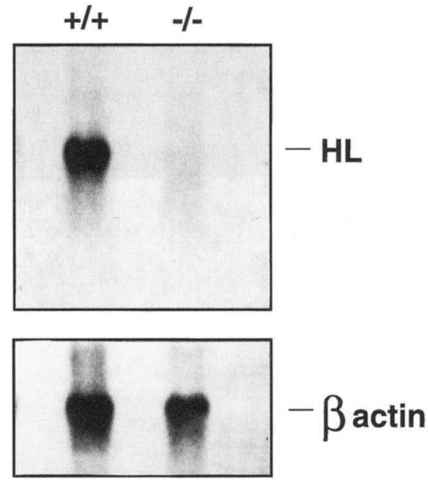

FIG. 2. **Northern blot analysis of liver mRNA.** Poly(A)$^+$ RNA (10 μg) isolated from liver of wild type and homozygous mutant mice were electrophoresed, blotted, and hybridized to a rat cDNA for HL as a probe. Judging from the rehybridization of the filter to an β actin probe, the amount of total mRNA in the +/+ lane was approximately twice of that in the −/− lane.

are small, the orientation of the *neo* gene in the HL gene did not appear to affect the overall targeting frequency. All five lines gave the expected size restriction fragments upon Southern analysis after digestion of their DNA with at least three different enzymes (data not shown). One of the three cell lines in which the *neo* gene is oppositely oriented from the transcription of the HL gene was used to generate mice lacking HL. Northern blot analysis of mRNA isolated from liver of homozygous mutants showed no significant hybridization to the rat HL cDNA probe, indicating the absence of any detectable transcripts for the HL gene (Fig. 2), whereas the liver of wild type mice contained mRNA for HL.

Plasma HL activity was assayed using a procedure specific for HL. Activities (nanomoles of free fatty acid released per min/ml ± S.D.) in wild type, heterozygous, and homozygous mice were 86.3 ± 19.4, 44.2 ± 6.6, and 1.8 ± 0.8, respectively ($n = 6$). All pairwise comparisons are significant ($p < 0.0005$). Thus, our targeted modification of the HL gene successfully eliminates HL activity in the mice.

Plasma Lipid and Lipoprotein Profiles—Fasted plasma lipid levels of wild type mice, and of mice heterozygous and homozygous for the inactivated HL gene, are listed in Table I. In both female and male homozygotes, plasma levels of total cholesterol are significantly increased by about 30% compared with the wild type mice. HDL cholesterol levels are also increased in homozygotes, although the value reaches statistical significance only in females. Since the ratios between HDL cholesterol and total cholesterol are the same in animals of all genotypes, the cholesterol carried by both HDL and apoB containing particles is increased in the homozygous HL deficient mice compared to wild type mice. Phospholipids are also significantly increased in homozygotes, but plasma levels of triglycerides are not altered in mutants compared with wild types.

In order to characterize the alterations in lipoprotein particles, plasma lipoprotein of different densities were separated by sequential ultracentrifugation. Plasma was collected in the morning from nonfasted normal and homozygous mutant mice fed regular chow. Analyses of the density fractions by agarose gel electrophoresis revealed two important differences in the plasma of the homozygous mutants compared with that of wild type mice (Fig. 3).

The first and most striking difference is an increase in the $d = 1.02–1.04$ density fraction in the homozygous mutants. This fraction contains β-migrating LDL, slow β-migrating apoB48-containing remnants, and α-migrating HDL (HDL_1). The lipoprotein particles migrating in this band react strongly with an antibody against apoE (Fig. 3*c*), but moderately with an antibody against apoA-I (Fig. 3*d*). They do not react at all with anti-apoB (Fig. 3*b*). We conclude that these HDL_1 particles are apoE rich and lack apoB. The increase of immunoreactive apoE-rich HDL_1 in the $d = 1.02–1.04$ g/ml fraction is consistent with an increase in the mass of apoE in this fraction that we observed in SDS-polyacrylamide gel electrophoresis analysis of the same lipoprotein fractions (Fig. 4; note twice as much sample of each density fraction from normal animals as that from mutants was loaded on this gel). Accumulation of these large HDL particles in the plasma of the mutants is consistent with previous findings that HL plays a role in the conversion of larger HDL into smaller HDL particles.

The second difference is in the amount of β-migrating particles in density range 1.10–1.21 that do not stain with Fat Red 7B (Fig. 3*a*), but react strongly with antibody against apoE (Fig. 3*c*) and moderately with antibody against apoA-I (Fig. 3*d*). These particles are decreased in the mutants compared in the wild type mice. This lipid-poor and β-migrating HDL in normal mouse plasma was described previously by de Silva *et al.* (30) as a potential precursors for larger HDL particles similarly to human pre-β-HDL particles, although its precise origin and function remain to be determined.

The third notable difference is in the size of apoB containing particles. Although the plasma of chow fed normal animals contains small particles carrying apoB of density higher than $d = 1.04$, these small apoB-containing particles are reduced and not detectable in the plasma of chow fed homozygotes (Fig. 3*b*). The presence of smaller apoB containing particles in normal mouse plasma and their co-elution with HDL particles in Superose 6 FPLC have been described (30). We infer from these results that in mice conversion of IDL to LDL is not affected by the lack of HL, although further processing to smaller LDL may be affected. On the other hand, there is no indication that LDL particles accumulate preferentially in the mutant mice,

TABLE I

Plasma lipid profiles in mice deficient in hepatic lipase

Plasma was collected from mice with mixed genetic background between 129 and C57BL/6J. Mice were between 2 and 8 months of age. Plasma levels of total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TG), and phospholipids are expressed as mg/dl, mean ± S.D. The Student's *t* test of unpaired observations was used to determine statistical significance against wild type levels. Phospholipid content was measured on five wild type, nine heterozygous and seven homozygous animals. +/+, wild type; +/−, heterozygotes; −/−, homozygotes. ND, not determined.

Animals	TC	HDL-C	HDL-C/TC	TG	Phospholipid
Females					
+/+ (n = 25)	93 ± 18	81 ± 20	0.9 ± 0.1	61 ± 17	170 ± 34
+/− (n = 28)	104 ± 21[a]	86 ± 20	0.8 ± 0.1	73 ± 24[a]	188 ± 45
−/− (n = 26)	126 ± 23[b]	98 ± 24[a]	0.8 ± 0.1	70 ± 32	208 ± 60[a]
Males					
+/+ (n = 11)	108 ± 15	97 ± 13	0.9 ± 0.1	77 ± 28	ND
+/− (n = 10)	115 ± 21	107 ± 24	0.9 ± 0.1	79 ± 20	ND
−/− (n = 11)	133 ± 25[c]	108 ± 27	0.8 ± 0.1[a]	77 ± 38	ND

[a] $0.005 < p < 0.05$.
[b] $p < 0.0005$.
[c] $0.0005 < p < 0.005$.

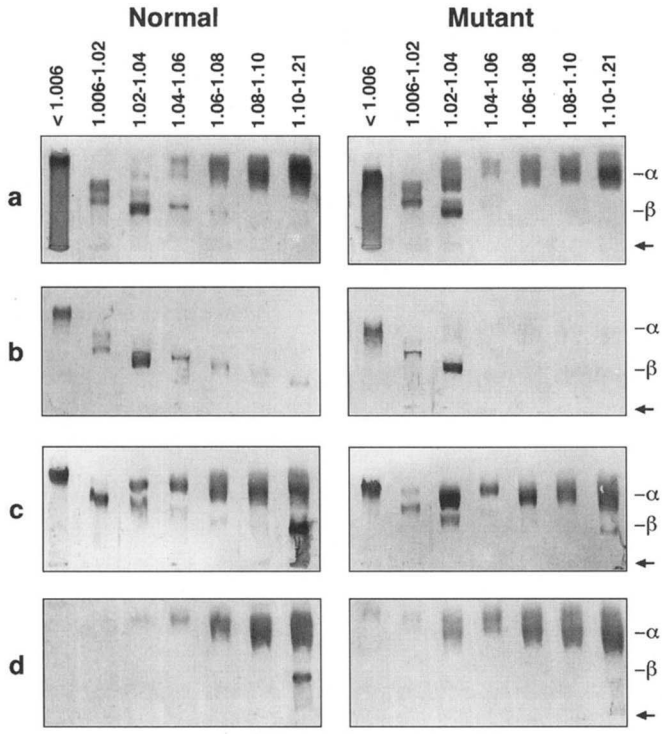

FIG. 3. **Agarose gel electrophoresis of lipoprotein fractions.** Equal volumes of plasma were pooled from six wild type mice and from six homozygous mutant mice fed regular chow, and plasma lipoproteins were separated by sequential density ultracentrifugation using the density ranges shown. Equal volumes of each fraction were loaded on a 1% agarose gel. Gels were stained with Fat Red 7B (*a*) or blotted to nitrocellulose and reacted with antibodies against human apoB (*b*), rat apoE (*c*), and mouse apoA-I (*d*). *Left panels* represent lipoproteins from wild type mice, and *right panels* represent those from mutant mice. The gel origins (*arrow*) and the positions of α- and β-migrating particles are indicated on the *right*.

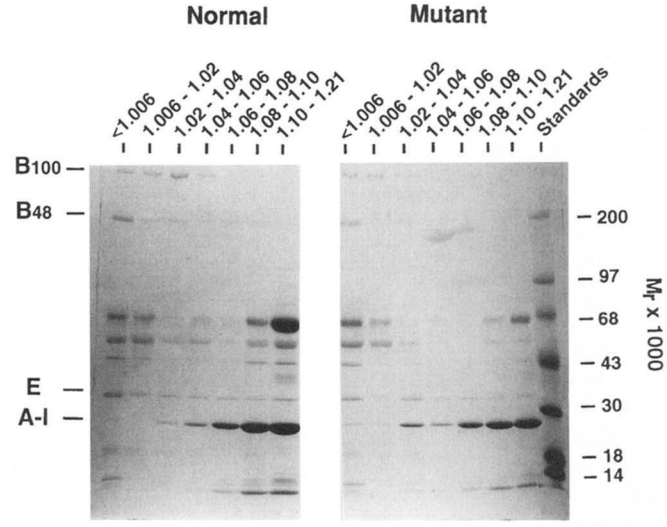

FIG. 4. **SDS-polyacrylamide gel electrophoresis of isolated lipoprotein fractions from chow-fed mice.** Aliquot of 30 μl for each density fraction from normal mice and 15 μl from mutants were applied to electrophoresis in denaturing 3–15% polyacrylamide gradient gels. Gels were stained with Coomassie Blue.

since the VLDL, IDL, and LDL fractions are all equally elevated.

The distribution of cholesterol, phospholipids, and triglyceride among lipoproteins in the various density fractions of chow-fed, nonfasted animals used in this experiment (n = 6) are shown in Fig. 5*a*. Plasma levels of cholesterol and triglyceride in the wild type mice were 75 ± 12 and 90 ± 9 mg/dl ± S.D. and in the mutant animals were 114 ± 25 and 110 ± 59 mg/dl ± S.D. Phospholipids recovered in the total lipoprotein fraction from 1 ml of plasma were 1.29 mg for normal mice and 2.99 mg for mutant mice. Although these data were derived from nonfasted animals and were using different reagent, the effect of genotype is the same as in the fasted animals described above (Table I). The distribution of cholesterol in the homozygous mutants was similar to that of wild type animals, except that in the mutants the fraction in the density range 1.02–1.04 contained 4.5 times more cholesterol compared with wild type. Since this fraction usually includes apoE-containing HDL particles, this result supports our observation of an increase in HDL_1 band seen in the agarose gel electrophoresis (Fig. 3). The phospholipid concentration in all fractions is increased in the mutants compared with the wild type animals. Triglycerides were mainly in particles of density range $d < 1.006$ in both normal and mutant mice.

In order to assess the effect of HL deficiency on the handling of high fat/high cholesterol diets, we fed animals an atherogenic diet containing 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate for 2 weeks. Normal mice and homozygous mutants both responded to the diet. Total plasma cholesterol increased by about 60 mg/dl; 159 ± 20 and 196 ± 5 (mg/dl ± S.D.), respectively, for normal mice (n = 4) and for homozygous mutant mice (n = 4). Plasma triglyceride levels in both normal animals and homozygotes are decreased slightly to 45 ± 14 and 67 ± 20 (mg/dl ± S.D.), respectively. Total phospholipid recovery in lipoprotein fractions was 2.26 and 5.00 mg/ml plasma from normal and mutant mice, respectively.

The distribution of lipids in the lipoprotein fractions from wild type animals and homozygous mutants fed the athero-

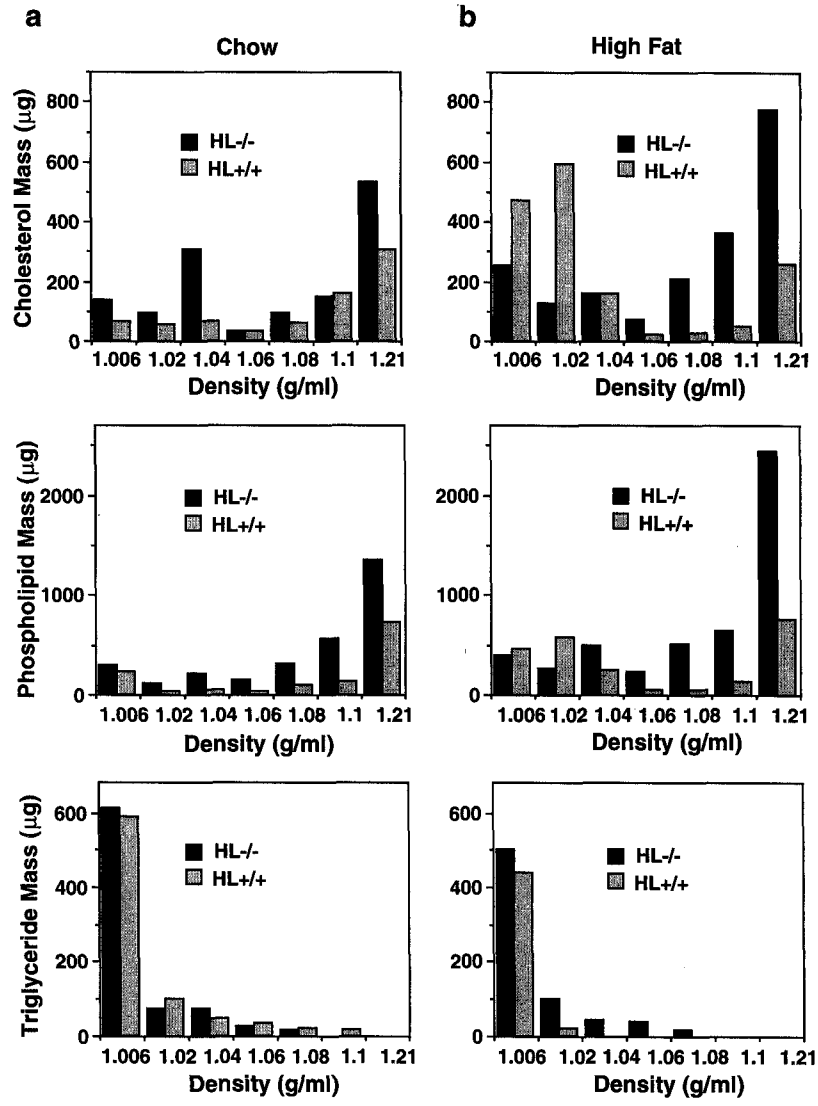

FIG. 5. **Lipid distribution among the lipoprotein fractions.** Total amount of cholesterol, phospholipids, and triglycerides in each density fraction from animals fed regular chow (*a*) or high fat diet (*b*) are shown. −/−, homozygotes; +/+, wild type animals. Values are average of two separate experiments and are adjusted for the total recovery.

genic diet are shown in Fig. 5*b* to compare with the distribution in chow-fed mice (Fig. 5*a*). Wild type animals responded to the high fat diet with an increase to about nine times normal cholesterol in the particles of density range $d < 1.02$ and with a decrease of cholesterol in particles of density range 1.06–1.21 to 60% of the level in chow-fed animals. The marked increase in the $d < 1.02$ fraction which contains VLDL and remnants, and the decrease in the $d = 1.06$–1.21 fraction which contains HDL, in response to the atherogenic diet of wild type mice is consistent with the earlier observations made by us and by others (37, 38). The changes in cholesterol distribution in the homozygous mutants in response to the atherogenic diet were quite different. Lipoprotein in the VLDL density range increased modestly to about 1.7 times normal. Cholesterol in $d = 1.06$–1.21 g/ml fractions increased to about twice that in chow-fed mutant animals (the cholesterol-carrying particles in these fractions are HDL). The distribution of phospholipids in mutants was very similar to that of cholesterol. No significant change in the distribution of triglycerides was seen in either normal or mutant animals in response to the dietary change. Overall these results again suggest that HL plays a major role in HDL metabolism.

Clearance of Triglyceride-rich Particles—Chylomicrons labeled with [^{3}H]cholesterol were isolated from the mesenteric lymph ducts of cannulated wild type mice and injected through the femoral vein of mutant and control mice (8–10 months old, males). At each time point, one animal was sacrificed and radioactivity remaining in plasma (Fig. 6*a*) as well as that recovered from liver was measured (Fig. 6*b*). The results show that the rates of disappearance of labeled cholesterol from plasma and its uptake by the liver did not differ between HL-deficient and control mice.

In order to test how mice lacking HL respond to acute fat loading, 5-month-old male mice were first fed 0.4 ml of olive oil as a bolus. Plasma triglyceride levels increased and peaked at about 2 h after loading in both mutants ($n = 5$) and control mice ($n = 5$) as shown in Fig. 7*a*; the area under the curves did not differ significantly between the mutants and controls. Thus there is no measurable difference in the response to this level of fat loading. When the mice ($n = 4$) were fed 1.0 ml of olive oil, initial clearance of triglyceride was scarcely impaired but mutants showed a second peak at 5 h that was not seen in the wild type animals (Fig. 7*b*). The majority of particles at 5 h after fat loading were in the fraction of $d < 1.006$, and the ratio of apoB100 to apoB48 (1:0.7) in these particles was similar to the ratio in the particles at 2 h. Our data do not allow us to decide the source of the 5-h particles. They could, for example, be chylomicrons made by delayed absorption of lipids by the intestine, or nascent VLDL particles packaged by the liver, or partially processed remnant particles released from the liver or other organs back to the circulation. Nevertheless, whatever the source of the 5-h particles, our results clearly show that

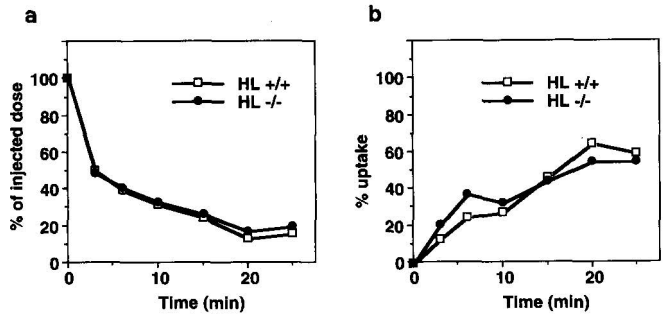

FIG. 6. **Clearance of exogenous mouse chylomicron by control and HL-deficient mice.** Chylomicrons labeled with [^{3}H]cholesterol were harvested from normal mice and injected into the femoral vein of control and homozygous mutant mice that had been anesthetized with nembutal. At the indicated times, 50 μl of blood was taken from the tail vein and radioactivity remaining in plasma was measured (*a*). Livers were perfused for 30 s with saline through the inferior vena cava and processed for determination of incorporated radioactivity (*b*). Each point corresponds to the measurement of a single animal.

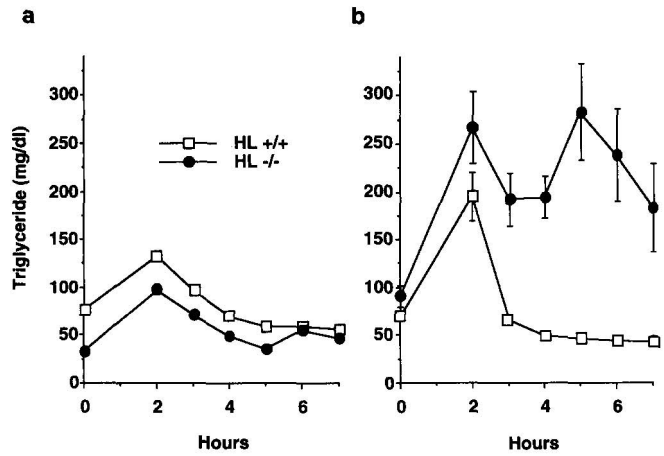

FIG. 7. **Response to acute fat loading in normal and HL-deficient mice.** Four males of each genotype received an intragastric administration of 0.4 ml (*a*) or 1 ml (*b*) of olive oil. Mean plasma triglyceride levels and standard errors of means are shown at different time points. At some points, the *error bars* are too small to show.

there is a difference between mice lacking HL and normal mice in their handling of triglycerides when the system is stressed to the extreme. On the other hand, the mutant mice can tolerate a modest level of loading without a major disturbance in their clearance of triglycerides.

Thus, although further detailed studies are necessary, results of our two experiments agree and suggest that the mutants do not have impairment in chylomicron and its remnant clearance.

DISCUSSION

We have generated mice lacking HL using gene targeting in mouse embryonic stem cells. Mutant mice lacking HL have elevated levels of plasma cholesterol, phospholipids, and HDL. These observations demonstrates *in vivo* the important role of HL in HDL metabolism and are consistent with the findings by Busch *et al.* (39) that transgenic mice expressing the human HL gene have reduced total plasma cholesterol and HDL cholesterol.

HDL in circulation exists in heterogenous forms. In humans, HDL_2 and HDL_3 are two discrete and stable classes of HDL particles both rich in apoA-I molecules, whereas HDL_1 particles are larger than HDL_2 and are rich in apoE but poor in apoA-I. In mice, HDL_2 and HDL_3 cannot be separated as discrete populations, but are particles of continuous sizes of density range d = 1.06–1.21. ApoE-rich HDL_1 particles in mice, on the other hand, are quite discrete in density range of d = 1.02–1.06, with a clear separation from apoA-I-rich HDL particles. We found that the mice lacking HL have elevated levels of HDL_1 particles compared with wild type litter mates when both are maintained on regular chow. The accumulation of HDL_1 in the mutants, together with our observation of their elevated plasma cholesterol and phospholipid levels, clearly demonstrate the importance of HL in the conversion of larger HDL particles to smaller particles. Thuren *et al.* (40) have shown that apoE-rich HDL are the preferred substrate for HL *in vitro*. The increase in HDL_1 seen when HL is absent suggests that this is also the case *in vivo*.

The heterogeneous HDL particles in circulation are thought to be generated and interconverted by processes intimately related to the metabolism of triglyceride-rich lipoproteins. Lipolysis of triglyceride-rich VLDL and chylomicrons by lipoprotein lipase has been shown to release excess surface coat, consisting of phospholipids, cholesterol, and apolipoproteins (41). These released components as well as nascent HDL proteins produced by the liver (42) become precursors for mature HDL. They quickly acquire cholesteryl esters and triglycerides through the function of lecithin cholesterol acyltransferase and cholesteryl ester transfer protein and eventually become larger HDL particles. At the same time, HL hydrolyzes excess triglycerides and phospholipids in the HDL particles which converts them into smaller size HDL particles. Our results showing that the conversion of HDL_1 to HDL_2 is prevented by absence of HL confirm the suspected obligatory role of HL in this process. Decrease of slow β-migrating HDL in d = 1.10–1.21 in mutant mice compared with wild type mice is compatible with these particles being the precursors of larger HDL or the products of hydrolysis.

We have shown that when mice lacking HL are challenged with a high fat/high cholesterol diet, the amount of cholesterol carried by particles in the density range d = 1.06–1.21 (mainly HDL_2 and HDL_3) doubles. On the other hand the amount of cholesterol carried by particles of d = 1.02–1.04 (mainly HDL_1) decreases in the mutant in response to the dietary change. ApoE increases in response to atherogenic diets (43), and apoE-rich HDL particles are cleared by LDL receptors and by receptors that recognize apoE (44). Possibly the clearance of HDL_1 through receptors that recognize apoE is enhanced in these mice. There is probably another pathway for the clearance of HDL that preferentially clears smaller apoA-I-containing HDL_3 particles (45). The increased level of HDL that we observed in mice lacking HL could be the consequence of a lack of surface phospholipid hydrolysis needed to remodel them into the smaller HDL particles preferred for clearance.

A surprising finding in our study is that mice lacking HL do not appear to have impairment in clearance of triglyceride-rich particles. Thus we found that the plasma TG levels for the mutants are not significantly increased over those in wild type mice and that the mutants clear chylomicrons from plasma at a rate equal to that in wild type mice. In addition, the mutants can tolerate acute fat loading as well as normal mice, unless the loading is extreme. Our observations appear to contradict the observations that treatments of rat liver with anti-HL antibody slow down clearance of chylomicrons from plasma (15, 16) and that humans with S267F plus T383M mutation have HL deficiency and delayed clearance of chylomicrons (46).

One of the likely explanations of these differences is that they reflect species differences. In mice, about two-thirds of HL function is in plasma, whereas in humans and rats HL is undetectable in plasma (47). Furthermore, mouse HL has a

lower affinity for heparin than does rat or human HL, and adult mouse liver has a significant amount of LPL activity, whereas there is little LPL activity in adult rat liver (47). These observations suggest that mice under normal conditions may depend more on LPL for facilitating clearance of triglyceride-rich particles by the liver than do humans and rats. In addition, very low levels of cholesteryl ester transfer protein function may influence the metabolism of triglyceride-rich particles in mice differently from that in humans.

The differences may also reflect the problems associated with the various experimental models. For example, antibody moiety interferes with the association of lipoproteins with receptors or with apoE on the vascular wall that are important for their clearance. Human HL deficiency may also be confounded by additional abnormalities.

We should also consider another and very plausible explanation for the differences between our observations and those in rats and in human patients. We suggest that *absence* of the HL protein has different effects from those associated with the *presence of nonfunctional* HL protein. Indeed, our mouse data lead us to predict that HL-deficient patients who completely lack HL protein will have near-normal function in terms of triglyceride metabolism. The mutant mice make no detectable HL message. The binding sites for HL in the livers of the mouse mutants are, therefore, most likely available for binding LPL which in turn can facilitate the clearance of chylomicron remnants. In contrast, nonfunctional HL may still bind to the cell surfaces and other proteins even though it has no catalytic activity. The *In vitro* transfection experiments using of Durstenfeld *et al.* (48) support this idea; they have shown that the human S267F and T383M mutant proteins are stable but have an extremely reduced enzyme function and that secretion of the mutant protein from the transfected cells is reduced to 12 and 20% of normal, respectively. It will be of interest to determine whether these secreted, but nonfunctional, mutant HL proteins bind to liver endothelium and prevent LPL from facilitating clearance of triglyceride-rich particles.

Some comment is required on our finding that the mice lacking HL can handle triglycerides almost normally, but that they have increased levels of HDL cholesterol and plasma phospholipids. LPL or other lipases are likely to be sufficient to maintain clearance of triglyceride-rich chylomicrons and VLDL in mutant mice lacking HL under ordinary conditions, because HL only poorly hydrolyzes the lipids in these particles which are the preferred substrates for LPL (49). We suggest that the clearance of triglyceride-rich particles occurs mainly because the lack of HL protein allows LPL to replace the function of HL in the liver. On the other hand, conversion of larger HDL into smaller HDL via phospholipid hydrolysis is unlikely to be efficiently compensated for in the mutants by LPL or other lipase activities in mutants, because LPL has much lower phospholipase activity than HL. This can explain why the mutants have elevated HDL cholesterol and phospholipids.

In conclusion, our mice lacking HL are useful for elucidating the complex pathways involved in the metabolism of HDL and triglyceride-rich particles and for investigating their interactions *in vivo*. Our suggested possibility that the consequence of total absence of the gene product could differ from the presence of nonfunctional gene product is not limited to the HL gene. This concept should be broadly considered when interpreting the results of various experiments where gene targeting is used to generate mutations in mouse genes.

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